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(54) Title: DIFFERENTIAL GENE EXPRESSION IN SCHIZOPHRENIA

(57) Abstract: Evidence for up-regulation of apolipoprotein L1, L2 and L4 gene expression in the prefrontal cortex of schizophrenia brains is presented. Methods of diagnosis of schizophrenia and methods of identifying compounds with potential activity in the prevention, treatment, or amelioration of schizophrenia are described.

Differential Gene Expression in Schizophrenia

This invention relates to diagnosis of schizophrenia, to methods of identifying compounds with potential activity in the prevention, treatment or amelioration of schizophrenia, and prevention, treatment or amelioration of schizophrenia.

Schizophrenia is a common disorder with a worldwide lifetime prevalence of approximately 1%, which typically has its onset in early adulthood and so interferes with education, employment and social functioning. Although there has been a long-running dispute over the psychological versus biological origins of schizophrenia, it is now accepted that it is, at least in part, a biological/ genetically determined disease. A hereditary contribution has been established beyond any doubt and there is a mounting body of evidence from structural neuroimaging and neuropathological studies for subtle brain abnormalities, both macroscopic and microscopic. Of all brain regions, the dorsal prefrontal cortex has been particularly implicated in the pathophysiological dysfunction in schizophrenia.

The sequencing of the human genome undoubtedly will open new exciting avenues for research into complex disorders such as schizophrenia. Furthermore, the availability of novel molecular profiling techniques such as microarrays and differential display (DD-PCR) techniques for the first time allow a global approach to screen for abnormalities in gene expression. These global approaches have the enormous advantage that they require no preconceptions (which are sometimes highly speculative and ill-founded as far as schizophrenia is concerned). Expression profiling techniques also have the benefit that no assumption has to be made as to whether the pathology is due primarily to genetic and/or environmental factors, as any causative effect will inevitably lead to changes in gene expression.

We have designed a candidate gene cDNA array to investigate changes in gene expression in the prefrontal cortex in schizophrenia and found a robust and reliable

change in expression of apolipoprotein L1 (apo L1), apolipoprotein L2 (apo L2), and apolipoprotein L4 (apo L4).

Lipoproteins are macromolecular complexes composed of lipids and proteins. They provide cells with lipids that are of vital importance to normal cell functioning. Lipids are used to generate energy, are building blocks for biomembranes, are essential for the synthesis of numerous lipophilic molecules (e.g. steroid hormones and Vitamin E) and play an important role in cell signalling and antioxidative mechanisms. Most lipoproteins are expressed in many tissues and cell types including the brain and cerebrospinal fluid (CSF). The apo L lipoproteins belong to the high density lipoprotein (HDL) family that play a central role in cholesterol transport. The cholesterol content of membranes is important in cellular processes such as modulating gene transcription and signal transduction both in the adult brain and during neurodevelopment.

According to the invention there is provided a method of diagnosing whether a subject has, or is at risk of developing schizophrenia, which comprises determining the expression level of the apolipoprotein L1, L2, or L4 gene in a biological sample obtained from the subject, or in a sample derived from a biological sample obtained from the subject.

The sequences of the apo L1 (SEQ ID NO: 1), apo L2 (SEQ ID NO: 2) and apo L4 (SEQ ID NO: 3) genes are shown in Figure 5.

The biological sample may comprise any of the following: CNS tissue, brain tissue, cells isolated from the prefrontal cortex, cells isolated from the developing neuroepithelium; a neural stem cell; or a progenitor cell.

It is possible that the level of apo L1, apo L2, or apo L4 expression in tissues other than brain tissue, which are therefore more easily obtained, may correlate with the level of expression of these genes in the prefrontal cortex. This can readily be established by comparing the expression level of the genes in different tissues to look for correlated

expression. It is expected that apo L1, apo L2, or apo L4 expression in cerebrospinal fluid, or peripheral tissue such as blood (especially leukocytes), epidermis, or nasal mucosa may correlate with apo L1, apo L2, or apo L4 expression in the brain.

Cells isolated from the developing human neuroepithelium can be isolated in culture and grown as aggregates termed neurospheres (Svendsen CN, and Smith AG, *Trends Neurosci* 1999 Aug; 22(8): 357-64). These contain a mixture of neural stem and progenitor cells, can be propagated in culture for extended time periods, and hold potential as a source of tissue for repairing the damaged CNS. According to the invention, the sample derived from the biological sample may be a neurosphere.

It is also possible that the expression level of the apo L1, apo L2, or apo L4 gene in the prefrontal cortex correlates with the level of a marker (which is not an expression product of one of these genes) in tissue other than brain tissue. Such tissue may be more readily accessible than brain tissue, thus facilitating diagnosis. The marker may be an expression product of a different gene. A marker with a correlated level of expression (a correlated marker) to the expression level of the apo L1, apo L2, or apo L4 gene can be identified simply by analyzing the expression levels of genes in different cell types of schizophrenic and non-schizophrenic subjects, and comparing the levels with the levels of expression of the apo L1, apo L2, or apo L4 genes in the prefrontal cortex of these subjects.

Thus, there is also provided according to the invention a method of diagnosing whether a subject has, or is at risk of developing schizophrenia, which comprises determining the level of a marker in a biological sample obtained from the subject, excluding brain tissue, wherein the level of the marker in the biological sample correlates with the expression level of the apolipoprotein L1, L2, or L4 gene in the brain. Preferably the biological sample is a peripheral tissue.

Methods of determining the expression level of a gene are well known to those of ordinary skill in the art. For example, the expression level may be achieved by

determining the level of mRNA or protein expressed from the gene in the biological sample.

Examples of suitable methods for determining the level of mRNA expression are quantitative PCR (in particular, real-time quantitative PCR) performed on cDNA produced by reverse transcription of the mRNA, and Northern blotting. Measurement of the concentration of apo L mRNA in several tissues by quantitative RT-PCR is described in Duchateau *et al* (2001) *Journal of Lipid Research* **42**, 620-630.

In a preferred method of determining the level of mRNA expressed from the apo L1, L2, or L4 gene, total RNA is obtained from the biological sample, cDNA synthesized from apo L1, L2, or L4 RNA present in the total RNA preparation, and the cDNA used for real-time quantitative PCR analysis to determine the level of apo L1, L2, or L4 mRNA in the sample. An embodiment of this method is described in detail in the Example below.

Examples of suitable methods for determining the level of protein expression are Western blotting and enzyme-linked immunosorbent assay (ELISA). Methods of quantifying levels of Apolipoprotein L in human plasma by competitive ELISA are described in Duchateau *et al* (1997) *Journal of Biological Chemistry* **272**, (41) 25576-25582, and in Duchateau *et al* (2000), *Journal of Lipid Research* **41**, 1231-1236.

A binding partner of an expression product of the apolipoprotein L1, L2, or L4 gene may be used to detect that expression product, for example to determine its expression level. The binding partner may be a protein, preferably an antibody or antibody fragment. The antibody or antibody fragment should bind specifically to a protein expression product of the apo L1, L2, or L4 gene so that the level of the protein expression product in the biological sample can be determined. Antibody to Apolipoprotein L is described in Duchateau *et al*, 1997, 2000, and 2001.

The binding partner may be a nucleic acid capable of hybridizing to a nucleic acid expression product of the apo L1, apo L2, or apo L4 gene. The nucleic acid should

hybridize specifically (for example under conditions of high stringency) to the nucleic acid expression product so that the level of the nucleic acid expression product in the biological sample can be determined. A preferred nucleic acid binding partner is an oligonucleotide primer for the synthesis of cDNA by reverse transcription from apo L1, L2, or L4 mRNA.

The level of a nucleic acid expression product of the apo L1, apo L2, or apo L4 gene is preferably determined by amplification of that nucleic acid expression product, for example by PCR. Thus, primers capable of amplifying the nucleic acid expression product are provided. Nucleic acid capable of hybridizing (preferably under conditions of high stringency) to nucleic acid that is complementary to a nucleic acid expression product of the apolipoprotein L1, L2, or L4 gene and/or nucleic acid which is a binding partner (preferably under conditions of high stringency) of an expression product of the apolipoprotein L1, L2, or L4 gene may be used to amplify a nucleic acid expression product of the gene, for example to detect an expression product of the gene.

Reverse transcription of apo L mRNA and PCR amplification of the resulting cDNA is described in Duchateau *et al*, 1997, and 2001.

There is also provided a kit for the diagnosis of schizophrenia that comprises a means for detecting an expression product of the apolipoprotein L1, L2, or L4 gene. The means for detecting the expression product may comprise a binding partner of an expression product of the apo L1, apo L2, or apo L4 gene, and/or a nucleic acid capable of hybridizing to nucleic acid that is complementary to a nucleic acid expression product of the apolipoprotein L1, L2, or L4 gene.

There is also provided according to the invention a method of diagnosing whether a subject has, or is at risk of developing schizophrenia, which comprises determining the level of an expression product of the apolipoprotein L1, L2, or L4 gene in the brain of the subject.

There is also provided according to the invention a method of prevention, treatment, or amelioration of schizophrenia in a subject which comprises reducing the level and/or activity of an expression product of the apolipoprotein L1, L2, or L4 gene in the brain, particularly the prefrontal cortex of the subject.

An inhibitor of the activity of an expression product could act by binding the expression product to inhibit its function, by inhibiting the interaction of the expression product with a binding partner required for the biological effect of the expression product, or by affecting the localization of the expression product so that it is not appropriately located to exert its effect. An inhibitor of the expression of the gene could act by inhibiting transcription or translation of the gene.

Nucleic acid capable of hybridizing to the apolipoprotein L1, L2, or L4 gene, or to a nucleic acid expression product of the gene, and thereby inhibiting expression of the gene is expected to have utility as a medicament, particularly for the prevention, treatment or amelioration of schizophrenia. Such nucleic acid may be encoded by a vector capable of directing expression of the nucleic acid in the brain, particularly the prefrontal cortex, of a subject.

The association of up-regulated apo L1, apo L2, or apo L4 gene expression with schizophrenia allows the use of an expression product of the apolipoprotein L1, L2, or L4 gene, a regulator of expression of the apo L1, apo L2, or apo L4 gene, or a binding partner of an expression product of the apo L1, L2, or L4 gene, as a target for drug discovery to try to identify compounds with potential activity for the prevention, treatment, or amelioration of schizophrenia.

According to the invention there is provided a method of identifying an inhibitor of the activity of an expression product of the apolipoprotein L1, L2, or L4 gene which comprises contacting the expression product in the presence and absence of a candidate inhibitor and determining the activity of the expression product in the presence and absence of the candidate inhibitor.

There is further provided according to the invention a method of identifying an inhibitor of the interaction of an expression product of the apolipoprotein L1, L2, or L4 gene with a binding partner of the expression product required for the biological effect of the expression product, which comprises contacting the expression product and its binding partner in the presence and absence of a candidate inhibitor and determining binding of the expression product to its binding partner in the presence and absence of the candidate inhibitor.

There is also provided according to the invention a method of identifying an activator of the interaction of an expression product of the apolipoprotein L1, L2, or L4 gene with a binding partner of the expression product which inhibits the biological effect of the expression product, which comprises contacting the expression product and its binding partner in the presence and absence of a candidate activator and determining binding of the expression product to its binding partner in the presence and absence of the candidate activator.

There is further provided according to the invention a method of identifying an inhibitor of expression of the apolipoprotein L1, L2, or L4 gene which comprises providing a system capable of expressing the gene, maintaining the system under conditions for expression of the gene in the presence and absence of a candidate inhibitor, and determining the expression level of the gene in the presence and absence of the candidate inhibitor.

Example

Novel molecular profiling techniques offer opportunities to investigate gene expression changes in complex neuropsychiatric disorders on a large scale. In this study we screened a custom-made candidate gene cDNA array comprising several hundred genes that have previously been either implicated in schizophrenia or make conceptual sense in the light of the current understanding of the disease, as well as a selection of genes located on

known high-susceptibility chromosome locations. The array screen using 10 schizophrenia and 10 control brains revealed that apolipoprotein L1 (apo L1), showed robust upregulation in the prefrontal cortex of schizophrenic individuals. This finding was cross-validated in a blinded quantitative PCR study on the Stanley Foundation brain set consisting of 60 well-matched tissue samples derived from the prefrontal cortex of 15 schizophrenic, bipolar, depressed and control individuals, confirming the significant upregulation of apo L1 gene expression in the schizophrenia group. Using quantitative PCR, expression profiles of other members of the apo L family (apo L2 – apo L6) were investigated showing that apo L2 and apo L4 were highly significantly upregulated in schizophrenia. Results were confirmed on an independent set of 20 schizophrenia and 20 control brains from Japan and New Zealand. Apo L proteins belong to the group of high density lipoproteins (HDL) and all 6 apo L genes are located in close proximity to each other on chromosome 22q12.

Results

A custom-made cDNA array consisting of over 300 schizophrenia candidate genes was screened with cDNA probes derived from the prefrontal cortex of 10 schizophrenia and 10 control samples taken from our post-mortem brain collection, a selection of high quality (intact mRNA signals) post-mortem tissue from Japan, New Zealand and Cambridge. We found that the apo L1 gene showed a robust upregulation of 2.6-fold overall in schizophrenia which was statistically significant ($p=0.03$) (Fig. 1). We went on to validate apo L1 expression blind using real-time PCR on the Stanley Foundation brain collection consisting of 60 brain samples comprising 15 samples in each group of schizophrenia, bipolar disorder, depression and normal control respectively. The breaking of the blind code by the Stanley Foundation showed that apo L1 was increased in the schizophrenia group by an average of 1.85 fold which was highly statistically significant ($p=0.01$) (Fig. 3a, Table 1 and 2). In a next step we validated apo L1 expression on a further 20 schizophrenia and 20 control samples from our own brain collection (these include the 10 schizophrenia and control samples from the array screen). This brain collection is less well matched, but we could confirm a significant 1.4 fold increase of

apo L1 expression in schizophrenia (p=0.04), however real-time PCR values showed overall more variability, particularly in older individuals (Fig. 3b).

As the apo L1 gene is part of a related gene family that is arranged in a cluster on chromosome 22q12.3 (Fig. 2), we decided to establish the expression levels of the other 5 apo L genes (apo L2 – apo L6). We performed real-time PCR assays on the Stanley Foundation samples, comparing expression levels in the 15 schizophrenia brains with the normal controls. Interestingly, the apo L2 and apo L4 were also highly significantly upregulated at 2.4 fold (p=0.0004) and 2.8 fold (0.009) respectively (Fig. 3a, Table 1 and 2). The extremely significant upregulation of apo L2 was validated on our own brain collection of 20 schizophrenia and 20 control brains, giving a 2.1 fold increase of apo L2 (p=0.008) (Fig. 3b). It is worth noting that the apo L1 and apo L2 genes are highly homologous (82% sequence and 67% aminoacid identity respectively) and are expressed at high levels (compared to GAPDH), whereas apo L4 transcripts are relatively less abundant in the prefrontal cortex. It has previously been reported that some of the apo L genes are expressed in the brain, however, the cellular distribution has not been investigated so far. We therefore examined the expression patterns of L1 and L2 using *in situ* hybridisation on control tissue showing a pan-neuronal expression in the human prefrontal cortex (Fig. 4).

Methods

Tissue collection

Fresh-frozen prefrontal cortex tissue obtained from the Stanley Foundation Neuropathology collection, as well as a collection of schizophrenia and control brains from Japan and New Zealand (NZ) were used in this study: the Stanley Foundation brain collection consisting of well-matched prefrontal cortex tissue derived from 15 schizophrenia, bipolar, depressed and control individuals and the NZ/Japan collection consisting of 20 schizophrenia and 20 matched control brains. For all cases, tissue was collected from patients who had previously been diagnosed according to the DSM-IIIR or DSM-IV. The Stanley Foundation brain collection is well-matched for age, pH, sex, race,

side of the brain and post-mortem interval, the details of which have been described previously by Torrey *et al.* (2000) *Schizophr Res* **44,** 151-5. The NZ/Japan brain collection is matched for age, sex and post-mortem interval.

RNA extraction and cDNA synthesis

Total RNA was extracted from post-mortem prefrontal cortices of schizophrenia, bipolar, depressed and control brains using the Tri-reagent RNA extraction protocol (Sigma, UK) in conjunction with a mechanical homogeniser (Thermohybaid). Typical yields were 1 μg of total RNA per 1 mg of tissue. RNA sample concentrations were determined by UV spectrophometry (average $OD_{260/280}$ >1.8). RNA integrity was confirmed by direct visualisation of 18S and 28S rRNA bands following agarose gel electrophoresis. RNA samples (8μg) were incubated with 10 units of DNAse I (Roche, UK) at 37°C for 20 mins to remove residual DNA followed by inactivation at 65°C for 10 mins. RNA samples were further purified using a CHROMA SPIN™ column (Clontech, UK) according to the manufacturer's instructions. RNA samples (0.5μg) were reverse transcribed with AMV reverse transcriptase (Roche, UK) using random hexamers according to the manufacturer's instructions.

High-density filter array procedure

Gene expression profiles of 10 control and 10 schizophrenia brain samples were analysed by hybridisation screening of nylon membrane cDNA arrays with radioactively labelled first strand cDNAs. The arrays contained ten control probes and 300 IMAGE cDNAs complementary to mRNAs of noted or implicated importance in schizophrenia. The clones were selected from the UK-HGMP-MRC HuGen2 gene index and by sequence comparison versus the dbEST database (National Centre for Biotechnology Information; Bethesda, USA). Each cDNA was selected on the basis that it represented the most 3' end of a mRNA and lacked repeat sequences. cDNAs were amplified by PCR using the primers, IMAGE-M13F (5'-GTTTTCCCAGTCACGACGTTG-3') (SEQ ID NO: 4) and IMAGE-M13R (5'-TGAGCGGATAACAATTTCACACAG-3') (SEQ ID NO: 5). The identities of the cDNAs were verified by dye terminator cycle sequencing (Perkin Elmer). Control cDNAs included *Arabidopsis thaliana* cytochrome c554 gene (used for

hybridisation signal normalisation); *Arabidopsis thaliana* poly (A) sequences of 50, 60 and 90 bp respectively and the vector pT7T3D (negative controls); housekeeping genes actin and GAPDH. PCR amplified cDNAs were purified (Milipore) and diluted to a concentration of 0.5 μg/μl. cDNAs were denatured by the addition of NaOH to 0.2M and robotically spotted (Flexys, Genomic Solutions) in quadruplicate onto Hybond-N^+ membranes (Amersham Pharmacia Biotech). cDNAs were fixed onto membranes by UV crosslinking (70,000 μJ/cm^2). 25 μg of total RNA from each sample was reverse transcribed by 200 units of Superscript II-RT (Life Technologies) extending an oligo-$dT_{25}VN$ (8.3μM) primer in the presence of 0.55 μM [^{33}P]dATP. Each RNA sample was spiked with 2 ng of *Arabidopsis thaliana* cytochrome c554 mRNA. RNA was destroyed by alkaline hydrolysis and labelled cDNAs purified (QIAquick PCR purification columns, Qiagen). Prior to hybridisation labelled cDNAs were incubated with 17 μg of oligodA_{80} for 2.5 h at 42°C in an attempt to prevent hybridisation of poly (T) sequences to poly (A) tails of immobilised cDNAs. Hybridisation was for 48 hours at 42°C in 9.5ml of hybridisation buffer (Ultrahyb, Ambion). Arrays were washed sequentially in 2 x SSC, 0.1% SDS at room temperature (2 x 5 min), 2 x SSC, 0.1% (w/v) SDS at 42 °C (2 x 5 min), 0.1 x SSC, 0.1% (w/v) SDS at 42 °C (2 x 15 min) and exposed prior to scanning using the Typhoon 8600 variable mode imager (Amersham Biotech). To normalise for variation in robotic spotting each membrane was stripped and rehybridised with 5 ng of [^{33}P] dATP-labelled pBluescript II SK(-) vector. After image acquisition, hybridisation signals were quantified using ArrayVision (Image Research Inc.). Signal processing was subsequently performed using Bluescript vector data to correct for variation in the relative amount of DNA present at equivalent positions on different arrays. Variations in labelling, hybridisation, washing and duration of phosphor screen exposure of different arrays hybridisations were taken into account by reference to the control *Arabidopsis thaliana* cytochrome c554. Quantified data were expressed as absolute gene expression levels and were comparable within all RNA samples.

Real-time quantitative PCR

Real-time PCR was performed using a ABI Prism 7700 sequence detection system (PE Applied Biosystems, UK) according to the manufacturer's instructions. Reactions were

performed in a 25µl volume including diluted cDNA sample, 0.5µM primers, 2mM $MgCl_2$, Nucleotides, Taq DNA polymerase, and buffer included in the SYBR Green I Mastermix (PE, Applied Biosystems, UK). Diluted cDNA samples produced from 12.5ng total RNA were added to each well. A typical protocol took 2 hrs to complete allowing for the detection of multiple transcripts and replicates within a 96-well plate format. PCR cycling conditions were 50°C for 2 min, 95°C for 10 min, and 40 cycles of 94°C for 15s, 60°C for 1 min. SYBR Green I RT-PCR data was collected using the Sequence Detector Software (SDS version 1.6, Applied Biosystems). Amplification plots and predicted Ct values from the exponential phase of the PCR were exported directly into Microsoft Excel worksheets for further analysis.

Primers, control and standard curve generation

Several primer sets for each gene were designed to intron/exon boundaries (eliminating contaminating genomic DNA signals). This procedure has allowed us to compare independently the mRNA transcript level detected for the same gene. Primer sequences were designed using the Primer Express software (Version I, PE Applied Biosystems) according to the manufacturer's guidelines. In general, amplicons were between 70 and 120 nucleotides. Melting curve analysis and agarose gel electrophoresis allowed us to verify the specificity of PCR amplifications (data not shown). Standard curve assays were performed by measuring the mRNA transcript level obtained using a specific primer set from a control cDNA sample diluted at 2 fold intervals. A similar standard curve was performed for the housekeeping gene GAPDH. Therefore we could normalise the mRNA transcript level against GAPDH at each dilution termed (ΔCt). The relationship between ΔCt and log input mRNA concentration was linear for all primer sets analysed. The slope of the standard curve should be ≤ 0.1 for accurate mRNA transcript level determination. All standard curve and sample assays were performed in duplicate to improve the accuracy of mRNA transcript detection. No-template control assays (NTCs) were performed for each primer set used which produced negligible signal detection, typically 38-40 Ct's in value. RNA quality and quantity of each sample was assessed by normalising data with respect to the GAPDH mRNA transcipt level determined. All primer sets used for analysing diluted cDNA samples typically produced 20-30 Ct's on

average, whereas the GAPDH value was 18 Ct's within the main detection window 0-40 Ct's in value i.e. within the accepted detection window.

In situ hybridisation

In situ hybridisation was carried out as described by Herrero *et al.* (1996) *Brain Res Mol Brain Res* **42,** 149-55. In brief, tissue sections were fixed in 4% paraformaldehyde, rinsed in 0.1M phosphate-buffered saline (PBS) and dehydrated through 70, 80, 90 and 100% ethanol. Sections were then incubated with 2ng ^{35}S-labelled antisense oligonucleotides in 100µl hybridisation buffer (20% saline sodium citrate (SSC), 10% dextrane sulphate, 1 x Denhardt's solution, 50% deionised formamide, 400µg/ml denatured salmon testes DNA and 0.3% 2-mercaptoethanol) at 37°C for 16-18h. To demonstrate the specificity of the hybridisation, control sections were hybridised with 2ng ^{35}S-labelled probe plus 200ng (100-fold) unlabelled probe. Following hybridisation, slides were passed through three brief rinses in 1 x SSC, followed by three 30 min stringent washes in 1 x SSC at 55°C, with a final wash in 1 x SSC at room temperature for 1 h. All washes were contained 0.0001% 2-mercaptoethanol. Sections were rinsed in 300 mM ammonium acetate/70% ethanol and allowed to air dry. Dried sections were exposed to BioMax MR film (Kodak, USA) for 7 days.

For cellular transcript localisation slides were coated in Illford K-5 nuclear tract emulsion. After exposure for 3 weeks at 4°C in light-tight boxes, sections were processed in Illford phenisol developer for 5 minutes, washed in 2% chrome alum/2% sodium metabisuphite for 5 minutes, fixed in 30% (w/v) sodium thiosulphate for 10 minutes and finally rinsed extensively in dH_2O. Sections were then dried before being counterstained with methylene blue and coverslipped.

Statistical analysis

An unpaired Student's t-test (two-tailed) was used for both, cDNA array and real-time PCR data to detect significant changes in gene expression. Data transformation (log 10) was performed if the individual subject group data exhibited a non-normal distribution or

the standard deviations (SD's) between groups were significantly different as detected by F-test statistic.

Analysis of covariance (ANCOVA) was used to establish clinical diagnosis as the main effect and consider post-mortem delay, age, sex, brain pH and illicit as well as prescribed drug treatments (dose and drug type) as covariates. These analyses did not show any significant correlations with potential confounds for the observed gene upregulation for apolipoprotein L1, L2 and L4 respectively.

Conclusions

We report the consistent and reliable upregulation of three members of the apolipoprotein L gene family (apo L1, apo L2 and apo L4). Independent analyses using cDNA arrays followed by cross-validation with quantitative real-time PCR on tissue taken from the prefrontal cortex of altogether 35 schizophrenia and 35 control brains as well as 15 bipolar disorder and 15 depression brains demonstrated a reproducible upregulation of apo L1 and apo L2 in patients with schizophrenia. Analysis of covariance (ANCOVA) taking into account variables such as post-mortem delay, age, sex and illicit as well as prescibed drug (dose and drug type) treatment did not show correlation with the observed gene upregulation. Furthermore, bipolar disorder patients who have been treated with neuroleptics only showed marginally significant upregulation of the apo L2 gene by 1.6 fold ($p=0.05$) (Fig. 2), it is therefore unlikely that neuroleptic treatment is a confounding factor. Furthermore three of the schizophrenia patients in the Stanley Foundation have never been treated with neuroleptics (#), but still show highly significant upregulation of apo L1 and apo L2 (Table 2).

Interestingly, the apo L genes are clustered on chromosome 22q12 (Fig. 2). This chromosome region is of interest because the velocardiofacial syndrome (VCFS) is located close by on chromosome 22q11 (Fig. 2). About one third of VCFS cases experience schizophrenia-like psychoses. A recent study also reported a significant

association between catechol-o-methyltransferase (COMT) genotype and abnormal prefrontal cortical function; COMT is also located on chromosome 22q11 (Fig. 2).

The function of apo L proteins in the brain is unknown. Using *in situ* hybridisation we found that apo L1 and L2 show a pan-neuronal expression in the prefrontal cortex (Fig. 4). From blood investigations it is clear that apo L proteins are functionally associated with apo A1, the major protein of HDL and of importance in cholesterol transport (Duchateau *et al*, 1997). The central nervous system contains almost a quarter of the unesterified cholesterol present in the whole body. These sterols are largely located in two pools, namely the membranes of glial cells and neurons and in myelin sheaths. The cholesterol in the brain is almost exclusively synthesised *in situ* (Dietschy and Turley (2001) *Curr. Opin. Lipidol.* **12**, 105-112). Abnormalities in cholesterol turnover have been associated with Alzheimer's disease and Niemann-Pick disease. Alterations in genes and proteins associated with lipid metabolism have been associated with schizophrenia, e.g. changes in phospholipid content, fatty acid content and cholesteryl esters. Elevation of apolipoprotein D in the prefrontal cortex in both schizophrenia and bipolar disorder has been observed (Thomas *et al* (2001) *PNAS USA* **98**, 4066-4071). Also several myelin-related genes have been found to be abnormally expressed in the prefrontal cortex of schizophrenia (Hakak *et al* (2001) *PNAS USA* **98** 4746-4751). Thus, it is possible that abnormalities in cholesterol turnover or lipid metabolism, myelin-dysregulation, or elevation of apolipoprotein D may be linked to upregulated apo L1, apo L2, or apo L4 expression.

Lipoproteins also have a role in a wide variety of other biological functions. HDL has anti-viral activity and has been shown to inhibit HIV infectivity and virus-induced syncytia formation. Apo A1 has also been found to inhibit herpes simplex virus (HSV)-induced cell fusion at physiological concentrations and reduces viral spread. Apolipoproteins are engaged in the regulation of protease activity and calcium signaling and in intercellular signaling during embryonic development (Danik *et al* (1999) *Crit. Rev. Neurobol.* **13**, 357-407; Herz (2001) *Neuron.* **29**, 571-581; Gotthardt *et al* (2000) *J. Biol. Chem.* **275**, 25616-25624). Some lipoprotein receptors are known to act as receptors

for Reelin, a protein that plays an important role in cortical lamination and has been shown to be down-regulated in the prefrontal cortex in schizophrenia (Trommsdorff *et al* (1999) *Cell* **97**, 689-701). Lipoproteins also seem to play an important role in axonal growth. Distal axons of cultured sympathetic neurons internalise proteins and lipids from lipoproteins and transport them retrogradely to the cell body (Posses De Chaves *et al* (2000) *J. Biol. Chem.* **275**, 19883-19890).

It is known from a study investigating cytokine-induced gene expression changes in endothelial cells that apo L3 expression is upregulated by tumor necrosis factor α (TNFα) (Horrevoets *et al* (1999) *Blood* **93**, 3418-3431). TNFα is a pro-inflammatory cytokine that can exert neurotrophic and neurotoxic effects. This suggests a role of apo L in inflammatory processes. It is thought that TNFα or other proteins in the TNFα signal transduction and transcription pathway may regulate apo L1, apo L2, or apo L4 expression. Thus, modulation of the effect of TNFα or one or more proteins involved in the TNFα signal transduction and transcription pathway, or modulation of their expression, may be used to prevent, treat, or ameliorate schizophrenia. These proteins may be used as targets for drug discovery to try to identify potential compounds for such prevention, treatment, or amelioration.

Figure legends

Figure 1

Results of hybridisation screening of nylon membrane cDNA arrays with radioactively labelled first strand cDNA derived from control and schizophrenia prefrontal cortex RNA. The expression of Apo L1 (a) and the housekeeping gene actin (b) are highlighted. Additional differentially expressed genes were detected after normalisation procedures.

Figure 2 –

Schematic diagram showing the genomic organisation of members of the apolipoprotein L gene family (apo L1 to apo L6) on human chromosome 22q12.3. Note the proximity of the apo L cluster to the loci for the velocardiofacial syndrome (VCFS) and the catechol-o-methyltransferase (COMT) (see text).

Figure 3

(a) Relative mRNA fold differences in apo L gene expression (apo L1 - apo L6) in the prefrontal cortex of control, schizophrenic, bipolar and major depression patients as determined by real-time PCR on the Stanley Foundation brain collection. Note that the bipolar group shows upregulation of apo L1, apo L2 and apo L4 genes, which reaches significance for apo L2 gene expression. Significant differences are indicated by asteriks as determined by unpaired student's t-test (two-tailed). See Table 1 and 2 for details.

(b) Relative mRNA fold differences in gene expression in the prefrontal cortex of apo L1 and apo L2 determined in 20 schizophrenia and 20 control samples (Japan, New Zealand). Significant differences are indicated by p-values and asteriks as determined by unpaired student's t- test (two-tailed).

Figure 4

Autoradiogram (a) and low-power photomicrograph (b) illustrating the expression of apo L1 transcripts in the prefrontal cortex of a control individual. Apo L1 transcripts appear

to be expressed in all neurons at high levels. The apo L2 gene also showed a neuronal distribution of expression (not shown).

Figure 5

The sequences of the Apolipoprotein L1, L2 and L4 genes are shown.

Tables

Table 1

The Stanley Foundation brain collection comprising 15 well matched schizophrenia, bipolar, depression and control samples respectively was used to determine the mean percentage differences for each apo L gene (apo L1 – apo L6). p values are shown in italics and p values < 0.05 were considered significant for unpaired student's t-tests (two-tailed). NA denotes subject groups not analysed.

Table 2

The ΔCt value was derived by normalising mRNA transcript levels against the housekeeping gene GAPDH. Each ΔCt was produced by the mean values taken for two independent replicates. Note that a higher ΔCt value indicates lower expression levels (a ΔCt decrease of 1 corresponds to a 2-fold increase in gene expression) . The mRNA level value was obtained by the equation $2^{-\Delta Ct}$. The symbol # denotes schizophrenic patients who never received neuroleptic treatment.

Table 1 Fold differences of apo L gene expression in the Stanley Foundation brain collection: comparison of psychiatric subject groups with normal controls.

	SCHIZOPHRENIA		BIPOLAR DISORDER		MAJOR DEPRESSIVE DISORDER	
Gene	Fold Δ	p value	Fold Δ	p value	Fold Δ	p value
ApoL1	1.85	p=0.013 *	1.07	p=0.69	-1.07	p=0.69
ApoL2	2.37	*p=0.00004* ***	1.60	*p=0.05* *	1.53	*p=0.17*
ApoL3	1.30	*p=0.12*	NA		NA	
ApoL4	2.70	*p=0.009* **	1.40	*p=0.39*	1.08	*p=0.77*
ApoL5	1.25	*p=0.24*	NA		NA	
ApoL6	1.41	*p=0.12*	NA		NA	

Table 2 Real time PCR analysis of gene expression for apo L1 and apo L2 in individual control and schizophrenic subjects.

Apo L1

Control Group	ΔCt	mRNA Level (x 1000)	Schizophrenia Group	ΔCt	mRNA Level (x 1000)	
1	9.46	1.42	1	8.42	2.91	#
2	9.35	1.53	2	8.39	2.98	
3	9.56	1.32	3	9.93	1.02	
4	9.56	1.32	4	8.81	2.22	
5	8.79	2.26	5	7.70	4.81	
6	10.64	0.62	6	8.40	2.96	#
7	9.34	1.54	7	9.15	1.76	
8	10.22	0.83	8	7.84	4.36	#
9	10.85	0.54	9	8.84	2.18	
10	10.93	0.51	10	9.56	1.32	
11	9.49	1.39	11	9.11	1.81	
12	9.23	1.66	12	7.78	4.54	
13	8.99	1.96	13	9.09	1.83	
14	8.88	2.12	14	9.28	1.61	
15	9.07	1.86	15	8.76	2.31	
Av.	9.62	1.39	Av.	8.73	2.57	

Apo L2

Control Group	ΔCt	mRNA Level (x 1000)	Schizophrenia Group	ΔCt	mRNA Level (x 1000)	
1	10.49	0.69	1	9.02	1.92	#
2	10.76	0.57	2	10.03	0.95	
3	10.23	0.83	3	8.64	2.51	
4	10.82	0.55	5	9.71	1.19	
5	11.46	0.35	6	9.35	1.53	
6	10.45	0.71	4	8.92	2.06	#
7	11.84	0.27	7	9.25	1.64	
8	8.96	2.01	14	8.88	2.12	#
9	9.71	1.19	8	8.33	3.11	
10	9.16	1.74	9	8.67	2.45	
11	9.52	1.36	10	8.59	2.59	
12	10.60	0.64	11	8.68	2.43	
13	10.30	0.79	12	9.47	1.41	
14	11.06	0.47	13	9.96	1.00	
15	9.35	1.53	15	8.51	2.74	
Av.	10.31	0.91	Av.	9.06	1.97	

Claims

1. A method of diagnosing whether a subject has, or is at risk of developing schizophrenia, which comprises determining the expression level of the apolipoprotein L1, L2, or L4 gene in a biological sample obtained from the subject, or in a sample derived from a biological sample obtained from the subject.

2. A method according to claim 1 wherein the biological sample comprises any of the following: CNS tissue, brain tissue, cells isolated from the prefrontal cortex, cells isolated from the developing neuroepithelium; a neural stem cell; or a progenitor cell.

3. A method according to claim 1 wherein the sample derived from the biological sample is a neurosphere.

4. A method according to claim 1 wherein the biological sample comprises cerebrospinal fluid, or peripheral tissue in which the level of expression of the apo L1, apo L2, or apo L4 gene correlates with the level of expression of the corresponding gene in the prefrontal cortex.

5. A method according to claim 4 wherein the peripheral tissue comprises blood, leukocytes, epidermis, or nasal mucosa.

6. A method according to any preceding claim wherein determining of the expression level of the apolipoprotein L1, L2, or L4 gene involves amplification of a nucleic acid expression product of the gene.

7. A method of diagnosing whether a subject has, or is at risk of developing schizophrenia, which comprises determining the level of a marker in a biological sample obtained from the subject, excluding brain tissue, wherein the level of the marker in the biological sample correlates with the expression level of the apolipoprotein L1, L2, or L4 gene in the brain.

8. Use of a binding partner of an expression product of the apolipoprotein L1, L2, or L4 gene for the diagnosis of schizophrenia.

9. Use according to claim 8 wherein the binding partner is a protein, preferably an antibody or antibody fragment.

10. Use according to claim 8 wherein the binding partner is a nucleic acid capable of hybridizing to a nucleic acid expression product of the gene.

11. Use of a nucleic acid capable of hybridizing to nucleic acid that is complementary to a nucleic acid expression product of the apolipoprotein L1, L2, or L4 gene for the diagnosis of schizophrenia.

12. Use of an expression product of the apolipoprotein L1, L2, or L4 gene, a regulator of expression of the apolipoprotein L1, L2, or L4 gene, or a binding partner of an expression product of the apolipoprotein L1, L2, or L4 gene, as a target for drug discovery.

13. A method of identifying an inhibitor of the activity of an expression product of the apolipoprotein L1, L2, or L4 gene which comprises contacting the expression product in the presence and absence of a candidate inhibitor and determining the activity of the expression product in the presence and absence of the candidate inhibitor.

14. A method of identifying an inhibitor of the interaction of an expression product of the apolipoprotein L1, L2, or L4 gene with a binding partner of the expression product required for the biological effect of the expression product, which comprises contacting the expression product and its binding partner in the presence and absence of a candidate inhibitor and determining binding of the expression product to its binding partner in the presence and absence of the candidate inhibitor.

15. A method of identifying an activator of the interaction of an expression product of the apolipoprotein L1, L2, or L4 gene with a binding partner of the expression product which inhibits the biological effect of the expression product, which comprises contacting the expression product and its binding partner in the presence and absence of a candidate activator and determining binding of the expression product to its binding partner in the presence and absence of the candidate activator.

16. A method of identifying an inhibitor of expression of the apolipoprotein L1, L2, or L4 gene which comprises providing a system capable of expressing the gene, maintaining the system under conditions for expression of the gene in the presence and absence of a candidate inhibitor, and determining the expression level of the gene in the presence and absence of the candidate inhibitor.

17. A method according to any of claims 13, 14, or 16 in which an inhibitor is identified, or a method according to claim 15 in which an activator is identified.

18. A method of diagnosing whether a subject has, or is at risk of developing schizophrenia, which comprises determining the level of an expression product of the apolipoprotein L1, L2, or L4 gene in the brain of the subject.

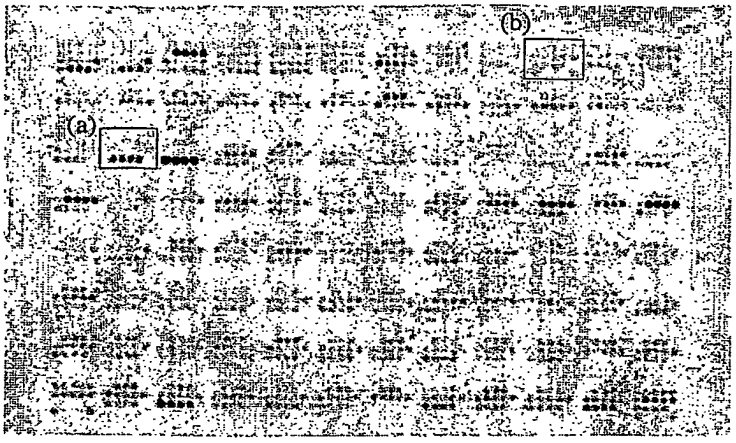
19. A method of prevention, treatment, or amelioration of schizophrenia in a subject which comprises reducing the level and/or activity of an expression product of the apolipoprotein L1, L2, or L4 gene in the brain of the subject.

20. A method of identifying a tissue in which the expression level of the apolipoprotein L1, L2, or L4 gene correlates with the expression level of that gene in prefrontal cortex tissue, which comprises: determining the expression level of the apolipoprotein L1, L2, or L4 gene in a tissue, other than prefrontal cortex tissue, of a schizophrenic and non-schizophrenic subject; and comparing the expression level in that tissue with the expression level of the gene in the prefrontal cortex of the subjects.

21. A method according to claim 20 wherein the tissue is cerebrospinal fluid, or peripheral tissue, such as blood, leukocytes, epidermis, or nasal mucosa.

22. A method of identifying a marker which is present in a tissue of a subject at a level which correlates with the level of expression of the apolipoprotein L1, L2, or L4 gene in the prefrontal cortex of the subject which comprises: determining the level of a candidate marker in a tissue, other than prefrontal cortex tissue, of a schizophrenic and non-schizophrenic subject; and comparing the level with the level of expression of the apolipoprotein L1, L2, or L4 gene in the prefrontal cortex of the subjects.

Schizophrenic



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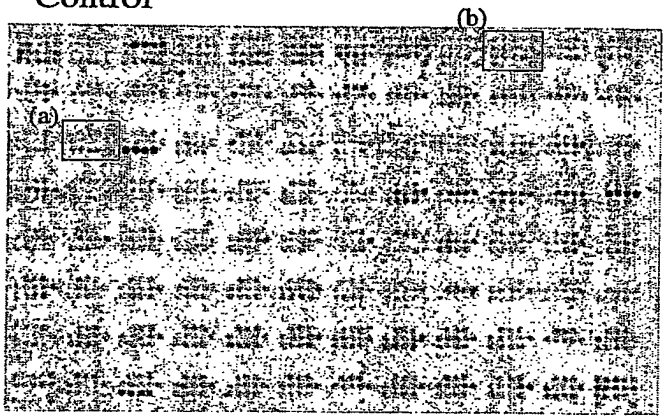

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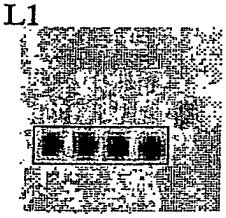

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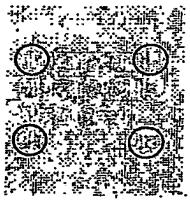


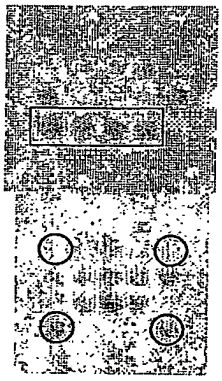
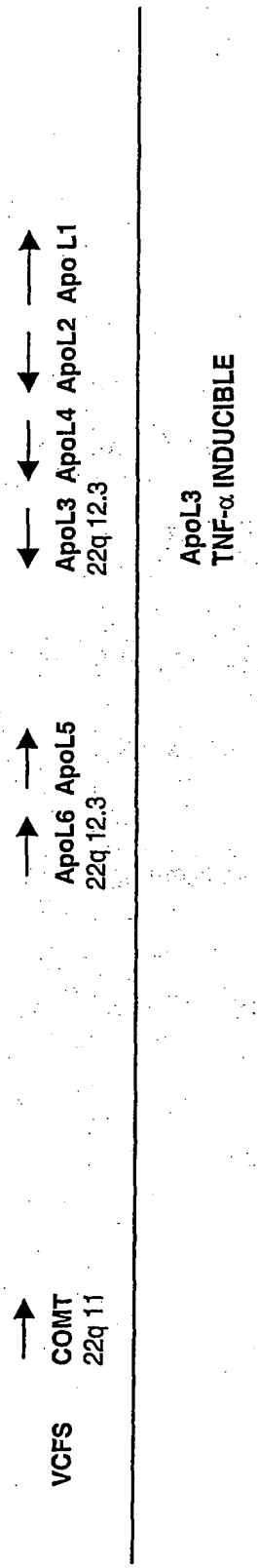


Figure 1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COMT
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22q 12.3
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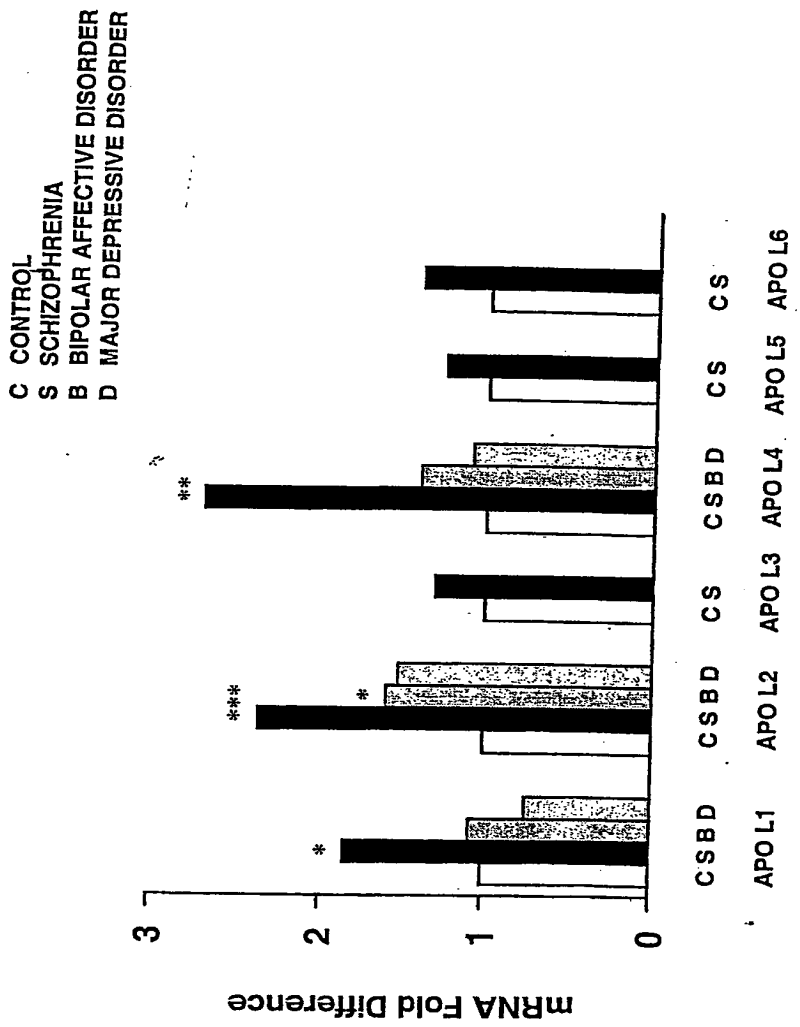
TNF-α INDUCIBLE

Figure 2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1
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3
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***
*
**
C S B D
APO L1
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APO L3
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APO L6

Figure 3a

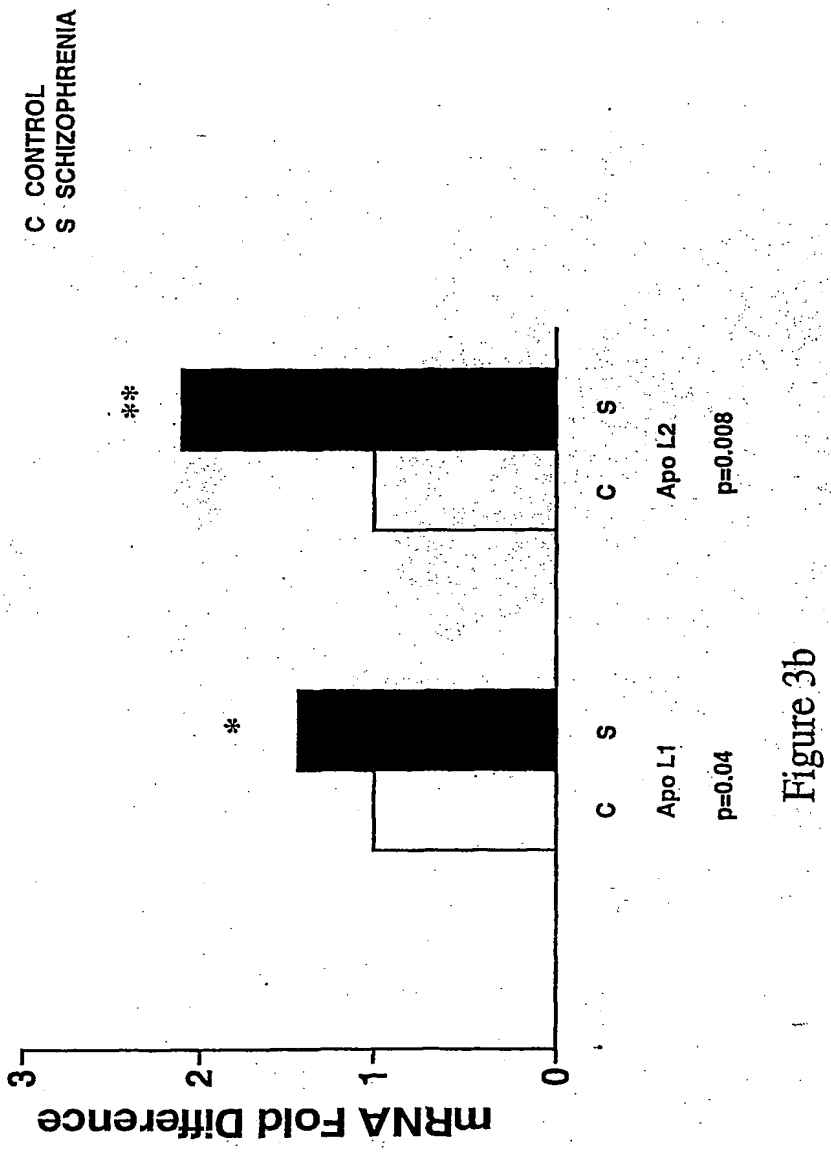
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Figure 3b

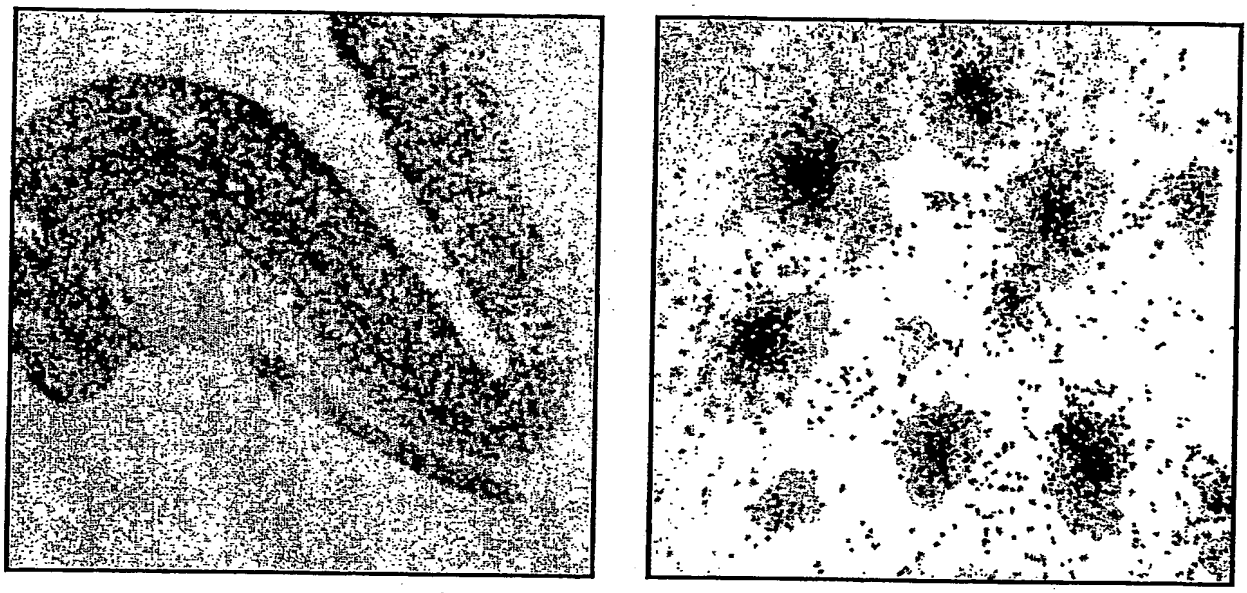

Figure 4

Figure 5 APOLIPOPROTEIN GENES - Chromosome 22q12.3

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GeneBank Acc No: AF019225

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Unigene No: 355475
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INTERNATIONAL SEARCH REPORT

International application No.

A. CLASSIFICATION OF SUBJECT MATTER

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.

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"P" document published prior to the international filing date but later than the priority date claimed

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"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
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0122789.1 21 September 2001 (21.09.2001) GB

(71) Applicant *(for all designated States except US)*: **THE BABRAHAM INSTITUTE** [GB/GB]; Babraham Hall, Babraham, Cambridge CB2 4AT (GB).

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(54) Title: DIFFERENTIAL GENE EXPRESSION IN SCHIZOPHRENIA

(57) Abstract: Evidence for up-regulation of apolipoprotein L1, L2 and L4 gene expression in the prefrontal cortex of schizophrenia brains is presented. Methods of diagnosis of schizophrenia and methods of identifying compounds with potential activity in the prevention, treatment, or amelioration of schizophrenia are described.

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(74) Agent: **THORNTON, Neil**; Reddie & Grose, 16 Theobalds Road, London WC1X 8PL (GB).

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(84) Designated States *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, SK, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

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(54) Title: DIFFERENTIAL GENE EXPRESSION IN SCHIZOPHRENIA

(57) Abstract: Evidence for up-regulation of apolipoprotein L1, L2 and L4 gene expression in the prefrontal cortex of schizophrenia brains is presented. Methods of diagnosis of schizophrenia and methods of identifying compounds with potential activity in the prevention, treatment, or amelioration of schizophrenia are described.

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INTERNATIONAL SEARCH REPORT

Interna Application No
PCT/GB 02/04285

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12Q1/68

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, WPI Data, PAJ, MEDLINE, BIOSIS, EMBASE, CHEM ABS Data, SCISEARCH, BIOTECHNOLOGY ABS, SEQUENCE SEARCH

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 01 46258 A (INCYTE GENOMICS INC ;AZIMZAI YALDA (US); KHAN FARRAH A (US); REDDY) 28 June 2001 (2001-06-28) SEQ-ID 47 page 13, line 14 - line 33 page 21, line 32 -page 22, line 2 page 32, line 31 -page 33, line 9 page 33, line 34 -page 34, line 5 page 53, line 19 -page 54, line 13 page 57, line 4 - line 29 page 58, line 7 page 59, line 15 - line 18 claims 5,11-15,27	8,10-12, 14,15

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

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16 July 2003	05/08/2003

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31–70) 340–2040, Tx. 31 651 epo nl, Fax: (+31–70) 340–3016	Bayer, A

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
X	WO 01 57275 A (CHEN WENSHENG ;HANZEL DAVID K (US); PENN SHARRON G (US); RANK DAVI) 9 August 2001 (2001-08-09) page 9, line 15 -page 10, line 18 page 11, line 10 - line 27 page 55, line 10 - line 13 page 59, line 16 -page 61, line 29 page 64, line 10 -page 65, line 23 page 66, line 10 -page 69, line 6 example 4 claims 1-22 ---	1-6,8, 10-18, 20,21
Y	PAGE N M ET AL: "The Human Apolipoprotein L Gene Cluster: Identification, Classification, and Sites of Distribution" GENOMICS, ACADEMIC PRESS, SAN DIEGO, US, vol. 74, no. 1, 15 May 2001 (2001-05-15), pages 71-78, XP004432227 ISSN: 0888-7543 the whole document ---	1-6, 8-11,18
Y	"A transmission disequilibrium and linkage analysis of D22S278 marker alleles in 574 families: further support for a susceptibility locus for schizophrenia at 22q12. Schizophrenia Collaborative Linkage Group for Chromosome 22." SCHIZOPHRENIA RESEARCH. NETHERLANDS 27 JUL 1998, vol. 32, no. 2, 27 July 1998 (1998-07-27), pages 115-121, XP002247905 ISSN: 0920-9964 abstract page 119, left-hand column, paragraph 2 -right-hand column, paragraph 1 ---	1-6, 8-11,18
Y	BASSETT A S ET AL: "Genetic insights into schizophrenia" CANADIAN JOURNAL OF PSYCHIATRY 2001 CANADA, vol. 46, no. 2, 2001, pages 131-137, XP009013978 ISSN: 0706-7437 page 133, right-hand column, paragraph 1 - paragraph 2 page 134, right-hand column, paragraph 2 page 135, left-hand column, paragraph 3 --- -/--	1-6, 8-11,18

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	DUCHATEAU PHILIPPE N ET AL: "Apolipoprotein L gene family: Tissue-specific expression, splicing, promoter regions;discovery of a new gene." JOURNAL OF LIPID RESEARCH, vol. 42, no. 4, April 2001 (2001-04), pages 620-630, XP002247906 ISSN: 0022-2275 cited in the application the whole document	1-6, 8-11,18
P,X	MIMMACK MICHAEL L ET AL: "Gene expression analysis in schizophrenia: reproducible up-regulation of several members of the apolipoprotein L family located in a high-susceptibility locus for schizophrenia on chromosome 22." PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF THE UNITED STATES OF AMERICA. UNITED STATES 2 APR 2002, vol. 99, no. 7, 2 April 2002 (2002-04-02), pages 4680-4685, XP002247907 ISSN: 0027-8424 the whole document	1-6,8-22

INTERNATIONAL SEARCH REPORT

Int nal application No.
PCT/GB 02/04285

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

see FURTHER INFORMATION sheet PCT/ISA/210

2. [X] Claims Nos.: 7 (complete); 8-15,17-19 (all partially)
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

see FURTHER INFORMATION sheet PCT/ISA/210

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box I.1

Although claim 19 is directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.
Although claims 8-11, 18 and claim 20-22 as far as in vivo is concerned are directed to a diagnostic method practised on the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

Continuation of Box I.2

Claims Nos.: 7 (complete); 8-15,17-19 (all partially)

Present claim 7 relates to a marker defined by reference to a desirable characteristic or property, namely that its level correlates with the expression level of apolipoproteins L1, L2 or L4 without giving any structural or essential characteristics of the marker.

Present claims 8, 12, 14, 15, 17 relate to a binding partner only characterized by its property of binding to an expression product of the apolipoproteins L1, L2 or L4.

Claim 12 relates to a regulator of expression characterized only by its property of regulation the expression of apolipoproteins L1, L2 or L4.

The claims cover all markers, binding partners or regulators having these characteristic or property, whereas the application provides support within the meaning of Article 6 PCT and disclosure within the meaning of Article 5 PCT for only a very limited number of binding partners and no support at all for markers or regulators. Thus, in the present case, the claims so lack support, and the application so lacks disclosure, that a meaningful search over the whole of the claimed scope for binding partners is impossible and no search for markers or regulators is possible. Independent of the above reasoning, the claims also lack clarity (Article 6 PCT). An attempt is made to define the markers, binding partners or regulators by reference to a result to be achieved. Again, this lack of clarity in the present case is such as to render a meaningful search over the whole of the claimed scope for binding partners impossible and no search for markers or regulators possible. Consequently, the search has been carried out for those parts of the claims which appear to be clear, supported and disclosed, namely those parts relating to antibodies to apolipoproteins L1, L2 or L4 and nucleic acids as binding partner in general (see e.g. present claims 9 and 10) whereas for the markers or regulators no search has been carried out.

Additionally claims 8-10, 12-14, 15, 17-19 relate to an expression product of the apolipoprotein L1, L2, L4 gene without further specification. Thus the claims cover all possible expression products (e.g. mRNA, proteins) whereas the application provides support within the meaning of Article 6 PCT and disclosure within the meaning of Article 5 PCT only for apolipoprotein L1, L2 or L4 mRNA (cDNA respectively). Thus the search has been carried out for apolipoprotein mRNA (cDNA) as expression product.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Furthermore present claims 10 and 11 relate to an extremely large number of possible nucleic acids since the nucleic acids are only defined by their hybridizing capability to apolipoprotein L1, L2 or L4 nucleic acids. No support within the meaning of Article 6 PCT and disclosure within the meaning of Article 5 PCT is to be found, however, for specific nucleic acids (defined by their essential characteristics, i.e. sequences). In the present case, the claims so lack support, and the application so lacks disclosure, that a meaningful search over the whole of the claimed scope is impossible. Consequently, the search has been carried on the general term "nucleic acid(s) capable of hybridizing" in connection with apolipoproteins L1, L2 and L4. Additionally a sequence search on apolipoprotein L1, L2 and L4 nucleic acids specified in SEQ-IDs 1-3 has been carried out.

The applicant's attention is drawn to the fact that claims, or parts of claims, relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure.

INTERNATIONAL SEARCH REPORT

Internal Application No
PCT/GB 02/04285

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
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